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Attenuation of the Cold-Adapted (ca) A/Krasnodar/101/35/1959 (H2N2) Influenza Strain: Role of the Ile147Thr Mutation in the PB1 Gene

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Kost V. [1,2], Tsfasman T. [1,3], Terekhov A.[1], Koptiaeva I.[1], Lisovskaja K.[1], and Markushin S.[1]

[1]Mechnikov Institute for Vaccines and Sera, per.Mal.Kazennij, 5a, 105064, Moscow, Russia

[2]Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry,16/10, Miklukho-Maklay str., 117997, Moscow, Russia,

[3]Institut Gustave Roussy, 39, rue Camille-Desmoulins, 94805 Villejuif, France

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ABSTRACT

Cold-adapted (*ca*) influenza virus strains used for live influenza vaccine production are attenuated in human and animal, which is probably due to their *ts* phenotype (no reproduction at 38-40^{o}C). We have previously obtained A/Krasnodar/101/35/1959(H2N2)*ca* strain designed for live influenza vaccine production, having 8 coding mutations acquired during cold adaptation in all the internal genes except NS. Here, in order to reveal genetic determinants of attenuation of this strain we have analyzed reassortants with one or more genes from the *ca* strain in the A/WSN/1933 strain genetic background, obtained via reverse genetics pHW2000-based system. Whenever NS or PB1 genes from the *ca* virus were included, the resulting reassortants displayed a *ts* phenotype, and reduced replication in mouse lung, unlike the parent A/WSN/1933 strain. Attenuation conferred by the NS gene of the *ca* strain to the reassortants was due to gene constellation effect. The role of the only coding mutation in the PB1 gene of the *ca* strain (Ile147Thr) was further assessed by site-directed mutagenesis. A reversion in the PB1 *ca* gene resulted in a complete reversion of phenotype of the respective single gene reassortant with A/WSN/1933. Inversely, introduction of Ile147Thr mutation into PB1 gene of A/WSN/1933 resulted in *ts* phenotype and a marked reduction of virus reproduction in mouse lungs. We conclude that the Ile147Thr mutation PB1 gene is the key determinant in *ts* and attenuated phenotype of A/Krasnodar/101/35/1959(H2N2) *ca* strain.

INTRODUCTION

Live influenza vaccines are currently in use in Russia and United States and represent a potent tool for influenza prevention. The vaccine is produced via reassortment between a cold-adapted (*ca*) attenuation donor strain and an epidemically relevant strain so that the reassortant contains surface proteins (HA and NA) from an epidemic strain and all the other proteins from the attenuation donor. Thus, the vaccine displays immunogenicity against the surface proteins of the epidemic strain and lack of virulence characteristic of the attenuated donor (Alexandrova§Smorodintsev, 1965; Ambrose *et al.*, 2008).

The two donor strains that are used for production of the influenza A vaccine component are the A/AnnArbor/6/60(H2N2) and A/Leningrad/134/17/57(H2N2) strains, in USA and Russia, respectively (Alexandrova§Smorodintsev, 1965; Ambrose *et al.*, 2008; Maassab, 1967; Maassab&Briant, 1999). Cold-adapted influenza strains used for live vaccine production have been adapted for replication at low temperature (25-26 ^{o}C, *ca* phenotype). This adaptation generally results in ts phenotype (inability to reproduce at 38-40 ^{o}C), and in reduced replication of the virus in respiratory tract of laboratory animals and humans (*att* phenotype).

The *ts*, *ca* and *att* phenotype of *ca* strains is dependent on the presence of mutations in at least some of the 6 viral RNA-segments, coding for the "internal" proteins (PB2, PB1, PA, NP, M1/M2 and NS1/NS2(NEP)). These mutations occur in the course of virus adaptation to low temperature, and probably have a cumulative effect on the viral phenotype. However, it has been shown on the model of A/AnnArbor/6/60(H2N2) and A/Leningrad/134/17/57(H2N2) strains, that some of these mutations play a more important role in the *ts* phenotype and attenuation of cold-adapted viruses, notably, mutations in the polymerase genes PB1 and PB2 (Jin *et al.*, 2003; Jin *et al.*, 2004; Isakova-Sivak *et al.*, 2011; Tsfasman *et al.*, 2007).

Although genetic determinants of *ts* and *att* phenotype have been thoroughly investigated for A/AnnArbor/6/60 (H2N2) and A/Leningrad/134/17/57(H2N2), there are still questions to answer in this domain. The exact parent strain of A/AnnArbor/6/60 cold-adapted virus remains unknown, and we do not know for sure if the attenuating mutations in this strain were acquired during low temperature passages. Therefore, it is unclear, whether attenuation related to polymerase genes is a general feature related to cold-adaptation of an influenza strain.

We have previously obtained a new A/Krasnodar/101/35/1959(H2N2) cold-adapted donor strain that underwent 35 passages at low temperature and was plaque purified in order to obtain an only clone of the virus (Ghendon *et al.*, 2013; Patent RF № 2354695). We have sequenced both the ancestor A/Krasnodar/101/1959(H2N2) strain and its cold-adapted mutant in order to identify mutations responsible for cold-adaptation (GenBank, JN596872-JN596877). During low temperature passages, the cold-adapted strain acquired 13 nucleotide substitutions in all the "internal" genes except the gene, coding for the NS1 and NS2 (NEP) proteins, 8 of them coding for an amino acid change in the PB2, PB1, PA, NP, M1, and M2 proteins. All the predicted amino acid changes were unique for the *ca* strain when compared to sequences of human influenza virus A strains from GenBank, which confirms their conservative nature. However, the role of individual coding mutations in the genome of A/Krasnodar/101/35/1959(H2N2) for the manifestation of its *ts*, *ca* and *att* phenotype remained unexplored.

In this study we have decided to access the mechanisms of attenuation of the *ca* A/Krasnodar/101/35/1959 strain obtained by our group, and to reveal, which genes are responsible for its *ts* and attenuated phenotype. To this end, we have obtained a panel of single gene and polygenic reassortants of the *ca* strain with a non-attenuated A/WSN/1933(H1N1) strain. Analysis of these reassortants and subsequent site-directed mutagenesis revealed the key role of PB1 gene in *ts* and attenuated phenotype of A/Krasnodar/101/35/1959 strain. These data are consistent with the results obtained previously on the model of A/AnnArbor/6/60 and A/Leningrad/134/17/57 cold-adapted strains. However, the Ile147Thr mutation was never before identified as a one that can confer *ts* and attenuated phenotype. Our approach permitted us to reveal a mutation in PB1 gene that could alone confer *ts* phenotype to a heterologous strain. These data could be beneficial for engineering potent live influenza vaccines from epidemic strains.

RESULTS

Generation of the reassortants between the A/WSN/1933 (H1N1) non-attenuated strain and the *ca* A/Krasnodar/101/35/1959(H2N2) strain

In order to investigate the role of every gene of the *ca*A/Krasnodar/101/35/1959(H2N2) in its attenuation we cloned all the genes of this strain into pHW2000. The insert compliance with the initial *ca* strain sequence were verified by sequencing. We observed no coding differences

between the initial and the reverse genetics produced virus. After that, we have obtained a panel of single-gene and polygenic reassortants with different combinations of genes inherited from the A/WSN/33 and A/Krasnodar/101/35/59 strains via reverse genetics. All the single-gene and polygenic reassortants obtained were viable and replicated well in allantoic cavity of chick embryos at 34 ^{o}C.

Single gene reassortants containing NS or PB1 genes from the *ca* strain display a distinct *ts* phenotype

Ts and *ca* phenotype of the reassortants obtained were assessed as virus titer in the allantoic cavity of chick embryos at 38 and 26 ^{o}C, respectively, compared to titers at optimal temperature (34 ^{o}C). The results were displayed as reproduction capacity at different temperatures (RCT_{38} and RCT_{26}), a difference in mean titers in lg EID_{50}/0.2 mlat the optimal and at a given temperature.

The initial A/WSN/33 strain had similar reproduction in chicken embryos at 34 ^{o}C and 38 ^{o}C and failed to grow at 25 ^{o}C. On the other hand, the *ca*A/Krasnodar/101/35/59 strain replicated well in chicken embryos at 34 ^{o}C, failed to replicate at 38 ^{o}C, and could replicate at 25 ^{o}C to high titers.

As could be seen from Table 1, only single gene reassortants containing PB1 gene or NS gene from the A/Krasnodar/101/35/59*ca* strain could not replicate at higher temperature (virus titre at 38^{o}C below 1.5 lg EID50/0.2 ml; RCT_{38}=5 and 6.5 EID_{50}/0.2 ml, respectively), so that their *ts* phenotype was similar to that of the initial *ca* strain. All the other reassortants on the A/WSN/33 genetic background carrying a single gene from the A/Krasnodar/101/35/59*ca* strain reproduced at 38^{o}C virtually to the same degree as at 34 ^{o}C (RCT_{38}<2 lg EID_{50}/0.2 ml), so that their phenotype did not vary from that of the initial A/WSN/33 strain.

Polygenicreassortants containing NS or PB1 genes from the *ca* strain display a distinct *ts* phenotype

Analysis of reassortants containing several genes from the *ca*A/Krasnodar/101/35/59 strain in the A/WSN/33 genetic background confirmed the results obtained on the single gene reassortants (Table 2). All the reassortants containing PB1 or NS gene had a marked *ts*

phenotype (no detectable virus at 38°C; RCT_{38}>5.5 lg EID_{50}/0.2 ml), unlike the reassortants containing other genes from the A/Krasnodar/101/35/59 and the initial A/WSN/33 strain.

It should be noted that neither of the A/Krasnodar/101/35/59 genes conferred *ca* phenotype to a single or polygenic reassortant (virus titers at 25°C below 1.5 lg EID_{50}/0.2 ml; RCT_{25}>4 lg EID_{50}/0.2 ml).

Reassortants containing NS or PB1 genes from the *ca* strain display reduced replication in mouse lungs

Replication of cold-adapted strains is known to be restricted to the upper respiratory airways of mice, which is one of the markers of their attenuation (Murphy&Coelingh, 2002). The restricted replication is due to the *ts* phenotype of cold-adapted strains and correlates with their attenuation in human respiratory tract.

In order to access the degree of attenuation of the reassortants obtained, we tested their ability to reproduce in mouse lungs and nasal turbinates. As was supposed, all the viruses reproduced well in upper airways of mice. However, single and polygenic reassortants containing the NS gene of A/Krasnodar/101/35/59 strain could not reproduce in mouse lungs, as well as the *ca* A/Krasnodar/101/35/59 strain itself (Tables 3 and 4). PB1 gene containing reassortants were partly attenuated and reproduced in mice to titers about 2 lg EID_{50}/1 g of lung tissue (compare to about 5-5.5 lg EID_{50}/1 g for A/WSN/33 and no detectable reproduction for the *ca* A/Krasnodar/101/35/59 strain).

It should be noted that PB1/PB2 double gene reassortant could not reproduce in mouse lungs.

Reversion of the Ile147Thr mutation in the PB1 gene of the *ca* strain results in *ts* and *att* phenotype reversion

As was determined before, during the low temperature passages the *ca* A/Krasnodar/101/35/59 strain acquired 13 mutations in all the internal genes (8 of them predicted to code for an amino acid change) (Ghendon *et al.*, 2013).

The only coding mutation in the PB1 gene occurred in position 147 (147 Ile→Thr; 464AUA→ACA). In order to understand the role of this mutation, we decided to reverse it in the PB1 gene of the *ca* A/Krasnodar/101/35/59 strain and to see the effect on the respective single gene reassortant with A/WSN/33 strain. Reversion from Thr to Ile was performed by

site-directed mutagenesis on the pHW2000 construct with the cDNA insert corresponding to the PB1 gene from the *ca* strain.

As could be seen from Table 5, the resulting PB1/WSN reassortant lost its *ts* phenotype in the allantoic cavity of chick embryos and reproduced at 38 ^{o}C to similar titers compared to 34 ^{o}C (RCT_{38}=0.5lg EID_{50}/0.2 ml). We also observed a non-attenuated phenotype of this reassortant in mouse lung model (titer in mouse lungs 5.0 lg EID_{50}/1 g of lung tissue, compared to 6.0 lg EID_{50}/1 g for A/WSN/33 strain and to undetectable titers for the *ca* A/Krasnodar/101/35/59 strain).

Ile147Thr mutation in the PB1 gene leads to attenuation of WSN virus strain

We have then introduced the Ile147Thr attenuating mutation in the PB1 gene of the A/WSN/33 strain by site-directed mutagenesis of the respective pHW2000/WSN construct and subsequent reverse genetics (Table 5). The resulting A/WSN/33 mutant acquired *ts* phenotype in embryonated chicken eggs (RCT_{38}=4.5lg EID_{50}/0.2 ml) and had a reduced replication in mouse lungs (3.0 lg EID_{50}/1 g of lung tissue, compared to 6.0 lg EID_{50}/1 g for A/WSN/33 strain and to 3.0 lg EID_{50}/1 g of lung tissue for single PB1 gene reassortant of *ca* strain in A/WSN/33 genetic background).

We conclude that the Ile147Thr mutation in the PB1 gene of the A/Krasnodar/101/35/59 *ca* strain is the key *ts* and *att* phenotype determinant for this strain. Furthermore, the attenuation conferred by this mutation could be transferred to a heterologous strain such as A/WSN/33 H1N1 strain.

It should be noted, that the NS gene of the A/Krasnodar/101/35/1959 *ca* strain did not have any mutations acquired during passages at low temperature. Therefore, the attenuation of the reassortants with this gene was due to other reasons, such as gene constellation between an H2N2 NS gene in the H1N1 genetic background (see Discussion).

DISCUSSION

This study was carried out in order to reveal genetic bases of the *ca* A/Krasnodar/101/35/1959 strain attenuation with a focus on the role of mutations in the "internal" genes of this strain in its *ts*, *ca*, and *att* phenotype. Similar investigations were conducted earlier on the model of the *ca* A/AnnArbor/6/60(H2N2), and

A/Leningrad/134/17/57(H2N2) strains (Jin *et al.*, 2003; Isakova-Spivak *et al.*,2011; Parks *et al.*,2007; Zhou *et al.*, 2012). It was shown that PB2, PB1, and NP genes were key determinants in the *ts*, *ca* and *att* phenotype of these strains.

In our model, any combination of genes that included the PB1 or NS genes of the A/Krasnodar/101/35/59 *ca* strain in the A/WSN/33 genetic background caused *ts* phenotype of a resulting reassortant, as well as its reduced replication in mouse lungs.

It should be noted that the NS gene of the *ca* strain unlike the PB1 gene did not have any mutations compared to the initial not attenuated and non-*ts* wild-type strain (this matter is discussed below). Therefore, we focused our attention on the mutations present in the PB1 gene of the A/Krasnodar/101/35/59 strain.

During passages at low temperature, the PB1 gene of the *ca* strain acquired only one coding mutation (Ile147Thr). Moreover, this mutation occurred at a conservative amino acid position in the PB1 gene. It was unique for the A/Krasnodar/101/35/59 cold-adapted strain compared to other epidemic and laboratory human influenza virus strains (Ghendon *et al.*, 2010), which suggests its possible role for the cold-adapted virus attenuation.

In order to access, if this mutation could have been responsible for the observed effects, we constructed a mutant of A/WSN/33 with Ile147Thr mutation in the PB1, and a reassortant containing mutated PB1 gene of the cold-adapted virus (Thr147Ile) in A/WSN/33 genetic background. Indeed, the 147th amino acid position in the PB1 proved to be crucial for the manifestation of *ts* phenotype of the reassortants obtained. Mutated A/WSN/33 strain did not reproduce at 38 ^{o}C in allantoic cavity of chick embryos and had reduced titers in mouse lungs, and was similar in its phenotype to the PB1 single gene *ca* strain/WSN reassortant. Inversely, reversion of the Ile147Thr mutation in the PB1 gene of the cold-adapted virus resulted in restored replication of the PB1 *ca* strain/WSN reassortant in mouse lungs and at high temperature in allantoic cavity of chick embryos.

We conclude that the Ile147Thr mutation in the PB1 gene can confer *ts* phenotype and reduced replication in animal respiratory tract to influenza viruses. This effect is common for different influenza virus strains, as A/Krasnodar/101/35/59(H2N2) strain and A/WSN/33(H1N1) strain, are genetically distinct, but the phenotype of both of them is influenced by this mutation.

As for the role of the NS gene of the *ca* strain in the A/WSN/33 genetic background, as noted previously, the *ca* A/Krasnodar/101/35/59 strain had no mutations in this gene compared to the corresponding wild-type virus. The wild-type A/Krasnodar/101/59(H2N2) progenitor strain containing the same NS gene as the *ca* strain did not display any of its attenuated properties. We conclude that the *ts* phenotype of the reassortant carrying the NS gene in the A/WSN/33(H1N1) strain background is primarily due to the gene constellation effects. It is interesting to note that the same pattern has been observed with a single-gene reassortant between the A/PuertoRico/8(H1N1) strain and A/Leningrad/134/57(H2N2) strain, containing the NS gene from the H2N2 strain and other genes from the H1N1 strain. This reassortant displayed *ts* phenotype unlike its wild-type parent strains (Egorov *et al.*, 1998). In both cases, this effect should be due to some amino acids that differ between the NS genes of the corresponding H2N2 strains and can cause an incompatibility between the NS1 or NS2/NEP proteins and other proteins in the H1N1 genetic background. We have made an *in silico* investigation of the candidate amino acids responsible for the *ts* phenotype of the reassortants. We have chosen amino acid positions that are conserved in all the H2N2 NS1 or NS2 proteins from the Influenza Database (NCBI) and that differ between both A/Leningrad/134/57 and A/Krasnodar/101/1959 strain from the one side, and A/Puerto-Rico/8-A/WSN/1933 strains from the other side. Thus we have found three candidate differences in amino acid sequence of the NS1 protein (Gln21Arg; Arg224Gly; Lys229Glu) and one candidate amino acid in the NS2/NEP (Phe107Leu). These genetic differences might account for the effect observed, and might be the source for possible incompatibility between the H2N2 NS1 or NEP proteins, and the polymerase complex of the H1N1 strain. However, a thorough mutant analysis is needed to prove this hypothesis.

It should be noted that transfer of a single gene from the A/AnnArbor/6/60 cold-adapted strain into the genome of a virulent A/Sydney/5/97(H3N2) strain did not lead to the *ts* and *att* phenotype of the latter (Parks *et al.*, 2007). Such effect could be achieved only by transferring at least two genes from the vaccine strain genome to the wild strain genome. In our experiments, the transfer of a single gene (PB1) of the cold-adapted strain to A/WSN/33 strain as well as the transfer of the respective Ile147Thr mutation in this gene to the PB1 gene of A/WSN/33 was sufficient for acquisition of *ts* phenotype by the resulting reassortant or mutant. However, this only mutation resulted in a decrease, but not in a complete abrogation of virus replication in mouse lungs. This speaks in favor of the cumulative role of other mutations in the cold-adapted strain for its attenuation. For instance, PB1/PB2 double gene

reassortant could not reproduce in mouse lungs and was more attenuated than other reassortants containing PB1 gene. This may account for cumulative role of mutations in cold-adapted A/Krasnodar/101/35/59 virus PB1 and PB2 genes. In this respect, A/Krasnodar/101/35/59 strain is similar to the well studied cold adapted vaccine donor strains A/Ann Arbor/6/60 and A/Leningrad/134/17/57. For both of these strains mutations in PB1 and PB2 genes were shown to be most crucial for the *ts* and *att* phenotype (Isakova-Sivak *et al.*, 2011; Jin *et al.*, 2003; Jin *et al.*, 2004). Notably, transfer of PB1 and PB2 genes of the cold-adapted A/Leningrad/134/17/57 strain into the genetic background of various epidemic strains conferred ts phenotype to these strains (Isakova-Sivak *et al.*, 2011).

Previous studies of attenuation mechanisms of other cold-adapted strains also showed the possibility to obtain attenuated mutant viruses by introduction of *ca* strain mutations into other strains, such as A/PuertoRico/8(H1N1) (PR8) strain (Jin *et al.*, 2004) or A/NewYork/1682/2009(H1N1) pandemic strain (Zhou *et al.*, 2012). For instance, mutations in PB1 gene of A/AnnArbor/6/60 PB1 (Lys391Glu, Glu581Gly и Ala661Thr) introduced into PR8 laboratory virus strain resulted in its *ts* and *att* phenotype.

The approach of introduction of attenuating mutations in heterologous strains can be also applied for production of genetically engineered attenuated vaccines on the basis of epidemical strains (Zhou *et al.*, 2012). Such vaccines could be more efficient than usual reassortant vaccines, because of additional cellular immune response for the internal genes of the vaccine virus. In the case of such genetically engineered attenuated vaccines, the immunogenic properties of all the internal genes would match those of a circulating epidemic strain.

We have thus identified a novel attenuating *ts* mutation in the PB1 gene of influenza viruses (Ile147Thr) that could be used for vaccine construction via reverse genetics technique.

Analysis of *ts* and *att* phenotype of polygenic reassortants indicates the dominance of genes responsible for the *ts* or *att* phenotype. Whenever PB1 gene from the *ca* virus was introduced into A/WSN/33 background, the resulting reassortant was *ts* and had a reduced replication in mouse lungs. This effect could not be rescued by any other *ca* genes that did not confer *ts* or *att* phenotype in respective single gene reassortants. This is consistent with data obtained by Parks *et al.*, 2007, who showed the dominance of attenuation conferred by A/AnnArbor/6/60 cold-adapted strain genes to reassortants with an epidemic A/Sydney/5/97(H3N2) strain.

These data confirm the safety of use of live influenza vaccines during an influenza epidemic when reassortants can be formed between epidemic and vaccine strains.

MATERIALS AND METHODS

Viruses

We used the A/WSN/1933(H1N1) strain, the wild-type A/Krasnodar/101/1959 (H2N2) strain, and the cold-adapted (*ca*) A/Krasnodar/101/35/1959(H2N2) strain. A/Krasnodar/101/35/1959(H2N2) strain is a donor strain designed for live influenza vaccines that have undergone serial passages at low temperature in chicken embryos and MDCK tissue culture and was cloned three times by plaque technique in MDCK cells. (Ghendon *et al.*, 2013).

Tissue culture

Madin-Darby canine kidney (MDCK) cells and 293T were maintained in minimal essential medium (MEM), supplemented with 10% fetal bovine serum (FBS). All cells were maintained at 37^{o}C, 5% CO_2.

Plasmids and bacteria

A pHW2000 plasmid and eight pHW2000-based plasmids containing all genes of the A/WSN/1933(H1N1) strain as inserts were kindly provided by Dr. Robert Webster (St Jude Children's Research Hospital, Memphis, USA). Plasmid DNA for recombinant influenza virus rescue was prepared using standard molecular cloning procedures. The E. coli DH5alpha strain was used for the propagation of plasmids. Chemically competent cells were prepared using the standard Hanahan method (Hanahan, 1983).

Propagation and concentration of viruses

The viruses were propagated in the allantoic cavity of the 9-day old embryonated eggs (34 ^{0}C, 48 h). The allantoic fluid from infected eggs was harvested and pooled. Virus stocks were kept at -80^{o}C.

For viral RNA isolation viruses were concentrated by centrifugation in Beckman J2-21 ultracentrifuge (rotor JA-14, 14000 rpm, 2,5 hours). Viral pellet was resuspended in STE – buffer (10mM Tris-HCL, 100mM NaCl, 1mM EDTA, pH 7,4).

Production of Krasnodar/WSN reassortants via reverse genetics

We used an eight–plasmid system for rapid generation of reassortant influenza A virus on the base of pHW2000 (Hoffmann *et al.*, 2002).

Generation of plasmids with the desired gene inserts of the cDNA corresponding to A/Krasnodar/101/35/59 cold-adapted virus was performed using standard techniques. Briefly, RNA was isolated from concentrated virus with the Viral RNA isolation kit (Isogen, Russia). Reverse transcription and PCR were performed according to manufacturer's specifications with M-MulV reverse transcriptase (Sibenzyme, Russia) and a high fidelity TersusPlus PCR kit (Eurogen, Russia), respectively. Terminal universal primer sequences for introduction of influenza virus genes into pHW2000 plasmid contained BsmBII (Esp3I) or BsaI(Eco31I) restriction sites and were provided by Dr.Robert Webster's group (St Jude Children's Research Hospital, Memphis, USA). Inserts were run on agarose gel and purified by the Silica Bead DNA Gel Extraction Kit (Fermentas, USA). Purified pHW2000 and inserts were simultaneously digested with appropriate enzymes (Esp3I or Eco31I for inserts, and Esp3I for the plasmid (Fermentas, USA)) overnight at 37^{o}C, dephosphorylated with Fast-AP phosphatase (Fermentas, USA) for 10 minutes, purified by DNA purification kit, ligated for 2h at 16 ^{o}C with T4 DNA ligase (Fermentas, USA), and transformed into E.coli by heat shock. Clones were selected on Amp Petri dishes, DNA was purified by Miniprep Kit (Eurogen, Russia), and sequenced in Eurogen (Russia), to confirm clone identity.

The method described by Hoffmann *et al.*, 2002 with some modifications was further used to rescue viable reassortant viruses. Briefly, 293T and MDCK 1:1 cocultured cells seeded a day before transfection were cotransfected with plasmids, containing various genes of the A/WSN/1933 and A/Krasnodar/101/35/1959 strains. Recombinant viruses were generated by DNA Lipofectamine-mediated transfection (Invitrogen) according to the manufacturer's specifications (http://www.invitrogen.com/etc/medialib/files/CellCulture/PDFs.Par.61792.File.dat/lipofectamineLTX and Plus _man.pdf). A non-transfected cell monolayer served as negative control. For a positive control, we transfected cells with a set of 8 plasmids, containing all the genes of the A/WSN/1933 strain. 48 hours after transfection the cells were treated with trypsin and were introduced along with the cell supernatant in allantoic cavity of chicken embryos for additional propagation of the reassortant viruses. With the reassortants that were easier to

rescue, we used MDCK cell culture for the last propagation step. The rescued viruses were aliquoted and stored at -80^{o}C.

Evaluation of ts and ca phenotype

Influenza virus titers were determined by viral reproduction assay in chicken embryos at different temperatures (34^{o}C, 38^{o}C and 25^{o}C). Virus titers were evaluated 48 h after incubation of chicken embryos at 34^{o}C and 38^{o}C, or after 6day incubation at 25^{o}C via Reed-and-Muench method. Viruses were detected via hemagglutination reaction with 0.5% erythrocytes. RCT (reproductive capacity at different temperatures) were evaluated as difference between titers at a given and at the optimal temperature. RCT_{25}=(lg EID_{50}/0.2 ml at 34 ^{o}C–lg EID_{50}/0.2 ml at 25 ^{o}C); RCT_{38}=(lg EID_{50}/0.2 ml at 34^{o}C – lgEID_{50}/0.2ml at 38^{o}C).

Viruses were considered as temperature-sensitive (*ts* phenotype), if $RCT_{38\text{-}40}$ was not less than 5.0 lg EID_{50}/0.2 ml, and cold-adapted, if RCT_{25} proves to be not more than 3.0 lg EID_{50}/0.2 ml.

Evaluation of pathogenicity of the viruses in mouse lungs

Evaluation of pathogenicity of the viruses was performed in the lungs and nasal turbinates of Balb/c mice as described earlier (Tsfasman *et al.*, 2007). Briefly, ether-anaesthetized mice (17-25 g) were infected intranasally with 0.04 ml of solution containing $10^{7.5}$ EID_{50}/0.2ml of the investigated virus strain. 72 hours after infection mice were sacrificed, and chicken embryos were infected with supernatant of 10 % suspensions of homogenized mouse lungs in RPMI medium. Virus titers in the 10 % suspensions were evaluated in allantoic cavity of chick embryos after incubation at 34 ^{o}C for 48 hours.

Site-directed mutagenesis of the PB1 gene

Site-directed mutagenesis was performed using standard methods with pre-designed mismatched primers and a two-step PCR with the use of a high fidelity DNA polymerase (TersusPlus PCR kit, Eurogen, Russia). The resulting mutated insert was introduced into pHW2000 as described above. Conformity of the construct with the desired sequence was accessed by sequencing (Eurogen, Russia). Primer sequences for site-directed mutagenesis were designed in the program written by our group. For mutated PB1 gene

A/Krasnodar/101/35/59 (GenBank accession JN596873) forward primer is K1T147IF: GCCAACACTATAGAGGTCTTCAG, reverse K1T147IR: AAGACCTCTATAGTGTTGGCTAGC. For mutated PB1 gene A/WSN/33 (GenBank accession CY034138) forward primer is W1I147TF: GCCAACACAACAGAAGTGTTCAGA, reverse W1I147TR: AACACTTCTGTTGTGTTGGCCAATG.

Sequencing

Sequence reactions were carried out by Eurogen, Russia, according to the Protocol for Fluorescent Sequencing with Big Dye Terminators (see http://www.bioinformatics.vg/Methods/sequencing.shtml) and were analyzed on a MegaBACE 500 analyzer (GF Healthcare). Gene sequences were viewed using the ChromasLite program (Technelysium).

Statistics

Statistical data processing was performed using the mean-square deviation.

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Table 1.Investigation of the *ts* and *ca* phenotype of single gene reassortants between the *ca* A/Krasnodar/101/35/1959(H2N2) and the A/WSN/1933(H1N1) influenza strains.

Parental viruses and reassortants	Genes inherited from the cold-adapted strain	Virus titer, lg EID_{50}/0.2 ml			RCT (reproduction capacity at different temperatures, difference in mean titers at a given and the optimal temperature)		Phenotype
		34°C	38 °C	25 °C	RCT_{38}	RCT_{25}	
A/WSN/1933	None	6.5±0.50	6.25±0.40	1.0	0.25	5.5	
A/Krasnodar/101 /35/1959	All	6.0±0.50	<1.0	5.5±1.0	6.25	0.75	*ts, ca*
1	PB2	5.0±0.50	4.5±0.50	<1.0	0.5	5	
2	PB1	6.5±0.32	1.5±0.40	1.5±0.40	5	5	*ts*
3	PA	6.25±0.25	6.5±0.28	1.5±0.40	-0.25	4.75	
4	HA	6.0±0.50	6.0±0.50	1.0	0	5	
5	NP	5.75±0.74	6.0±0.86	<1.0	0.25	5.75	
6	NA	5.0±0.40	5.5±0.50	1.0	-0.5	4	
7	M	4.75±0.25	3.75±0.2	<1.0	1.25	4.75	
8	NS	5.5±0.40	<1.0	1.5±0.57	6.5	5	*ts*

**Gene segments are highlighted in gray (A/Krasnodar/101/35/1959). The A/WSN/1933 segments are uncoloured.*

Table 2.Investigation of the *ts* and *ca* phenotype of polygenic reassortants between the *ca* A/Krasnodar/101/35/1959(H2N2) and the A/WSN/1933(H1N1) influenza strains.

Parental viruses and reassortants	Genes inherited from the cold-adapted strain	Virus titer, lg EID_{50}/0.2 ml			RCT (reproduction capacity at different temperatures, difference in mean titers at a given and the optimal temperature)		Phenotype
		34^0C	38^0C	25^0C	RCT_{38}	RCT_{25}	
A/WSN/1933	None	6.5±0.50	6.25±0.40	<1.0	-0.5	6.5	
A/Krasnodar/101/35/1959	All	6.0±0.50	<1.0	5.5±1.0	6.5	1	*ts*, *ca*
9	PB2, PB1, PA	6.5±0.24	<1.0	2.5±1.0	6.5	4	*ts*
10	PB2, NP	6.0±0.56	4.25±1.0	<1.0	1.75	6	
11	PB1, PA	6.0±0.50	1.0	1.5±0.40	5	4.5	*ts*
12	NP, NS	5.5±0.75	<1.0	<1.0	5.5	5.5	*ts*
13	NP, M	5.0± 1.0	3.75±0.75	<1.0	1.25	5	
14	M, NS	5.5±0.78	<1.0	<1.0	5.5	5.5	*ts*
15	PA, NS	5.5±0.75	<1.0	<1.0	5.5	5.5	*ts*
16	PB1, NS	5.25±0.20	<1.0	1.5±0.40	5.5	4	*ts*
17	PB2, PB1	5.5±0.75	<1.0	<1.0	5.5	5.5	*ts*

Table 3. Replication of single gene reassortants between the *ca* A/Krasnodar/101/35/1959(H2N2) and the A/WSN/1933(H1N1) influenza strains in the respiratory tract of mice (intranasal inoculation)

Parental viruses and reassortants	Genes inherited from the cold-adapted strain	Genome composition of the reassortants								Virus titer in nasal turbinates (lg EID_{50}/1.0 g)	Virus titer in lungs (lg EID_{50}/1.0 g)	Attenuated phenotype
A/WSN/1933	None	PB2	PB1	PA	HA	NP	NA	M	NS	5.5±0.50	5.16±0.56	
A/Krasnodar/101 /35/1959	All	PB2	PB1	PA	HA	NP	NA	M	NS	3.5±0.50	<1.0	*att*
1	PB2	PB2	PB1	PA	HA	NP	NA	M	NS	5.0±0.25	4.5±0.81	
2	PB1	PB2	PB1	PA	HA	NP	NA	M	NS	4.40±0.14	2.0±0.70	*att(+/-)*
3	PA	PB2	PB1	PA	HA	NP	NA	M	NS	4.5±1.0	3.5±0.91	
4	NP	PB2	PB1	PA	HA	NP	NA	M	NS	4.5±0.81	5.0±0.50	
5	M	PB2	PB1	PA	HA	NP	NA	M	NS	4.6±0.62	3.5±0.40	
6	NS	PB2	PB1	PA	HA	NP	NA	M	NS	3.0±0.70	<1.0	*att*

Table 4 Replication of polygenic reassortants between the *ca* A/Krasnodar/101/35/1959(H2N2) and the A/WSN/1933(H1N1) influenza strains in the respiratory tract of mice (intranasal inoculation).

Parental viruses and reassortants	Genes inherited from the cold-adapted strain	Genome composition of the reassortants								Virus titer in nasal turbinates (lg EID_{50}/1.0 g)	Virus titer in lungs (lg EID_{50}/1.0 g)	Attenuated phenotype
A/WSN/1933	None	PB2	PB1	PA	HA	NP	NA	M	NS	4.5±0.40	5.5±0.40	
A/Krasnodar/101 /35/1959	All	PB2	PB1	PA	HA	NP	NA	M	NS	3.5±0.70	<1.0	*att*
7	PB2, PB1, PA	PB2	PB1	PA	HA	NP	NA	M	NS	4.0±0.40	2.0±0.40	*att(+-)*
8	PB2, NP	PB2	PB1	PA	HA	NP	NA	M	NS	5.0±0.61	6.0±0.40	
9	PB1, PA	PB2	PB1	PA	HA	NP	NA	M	NS	5.0±1.22	2.0±1.10	*att(+-)*
10	NP, NS	PB2	PB1	PA	HA	NP	NA	M	NS	3.5±0.40	<1.0	*att*
11	NP, M	PB2	PB1	PA	HA	NP	NA	M	NS	4.25±0.53	4.75±0.24	
12	M, NS	PB2	PB1	PA	HA	NP	NA	M	NS	3.5±0.65	<1.0	*att*
13	PA, NS	PB2	PB1	PA	HA	NP	NA	M	NS	3.75±0.32	<1.0	*att*
14	PB1, NS	PB2	PB1	PA	HA	NP	NA	M	NS	3.75±0.93	<1.0	*att*
15	PB2, PB1	PB2	PB1	PA	HA	NP	NA	M	NS	3.25±0.53	<1.0	*att*

Table 5. Influence of the 147 Thr or Ile amino acids in PB1 gene on the *ts* and *att* phenotype of the A/WSN/33 and *ca* A/Krasnodar/101/35/59 strains.

Virus strain, mutant or reassortant	Titre at different temperatures, lg EID_{50}/0.2 ml *		RCT_{38}	Mean viral titer in mouse lungs on the 3th day after infection, lg EID_{50}/1.0 g*	Phenotype	PB1 gene characteristics	
	34 °C	38 °C				147 aa position	PB1 gene source
A/Krasnodar/101/35/1959 *ca* strain	7	<1	7	<1	*ts, att*	Thr	*ca strain*
A/WSN/33 strain	7	7	0	5.3	*ts+, non-att*	Ile	*A/WSN/33 strain*
PB1 ca/WSN reassortant	6.5	1.5	5	1.3	*ts, att (+-)*	Thr	*ca strain*
PB1 carevertant (Thr147Ile)/WSN reassortant	6	5.5	1.5	4.3	*ts+, non-att*	Ile	*ca strain*
A/WSN/33 mutant (PB1, Ile147Thr)	6.5	2	4.5	2.3	*ts, att (+-)*	Thr	*A/WSN/33 strain*

*This Table summarizes results of one representative experiment out of two.